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Europäisches Patentamt  
European Patent Office  
Office européen des brevets

11

Publication number:

**0 254 430  
A2**

12

## EUROPEAN PATENT APPLICATION

21 Application number: 87305669.1

22 Date of filing: 25.06.87

51 Int. Cl. 4: **G01N 33/543**, G01N 21/47 ,  
//G01N33/557,G01N33/76,G01-  
N33/78,G01N33/94,G01N33/80

The title of the invention has been amended  
(Guidelines for Examination in the EPO, A-III,  
7.3).

30 Priority: 26.06.86 US 879236

43 Date of publication of application:  
27.01.88 Bulletin 88/04

84 Designated Contracting States:  
**AT BE CH DE FR GB IT LI LU NL SE**

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54 **Light-scattering immunoassay system.**

57 A new immunoassay system is provided for the detection of ligands or ligand binding partners in solution in a heterogeneous format. The invention relies upon the detection of back scattered light from an evanescent wave disturbed by the presence of a colloidal gold label brought to the interface by an immunological reaction. The evanescent wave existing at the interface in turn is the result of a totally internally reflected incident light wave. Placement of the detector at a back angle above the critical angle insures a superior signal-to-noise ratio.

**EP 0 254 430 A2**

## SCATTERED TOTAL INTERNAL REFLECTANCE IMMUNOASSAY SYSTEM

Field of the Invention

This invention relates to immunoassays generally and more particularly provides a new system for performing virtual homogeneous immunoassays employing colloidal gold.

Background of the Invention

Many human disease states are identified on the basis of immunoassay techniques which rely upon the specificity between antibodies and antigens, or ligand binding partners and ligands respectively and interchangeably. Over the past fifteen or so years, there has been a substantial amount of effort involved in the development of immunoassay techniques utilizing the so-called sandwich and competitive techniques. The sandwich technique involves the immobilization of an antigen by one antibody and then subsequent labeling by attachment of a second antibody having associated therewith a detectable label. Reverse immunoassays for the detection of antibody are similar but instead put antigen on the surface for reaction with the sample antibody. Competitive techniques are useful for antigens having only a single epitopic site for reaction with an antibody. Accordingly, and as the name implies, such techniques rely upon the competition of the antigen with another labeled antigen for a binding site on an immobilized antibody. The substitutions necessary for antibody detection tests are obvious and need not be covered here in any great detail.

Of great importance in the laboratory is the development of highly sensitive techniques which can be run in either batch random access, panel, or stat modes. Preferably, such techniques will be homogeneous in nature, i.e., and as used herein, they will be conducted solely within one container without any accompanying requirement to physically separate out components following reactions during the assay.

It is one object of the present invention to provide a new immunoassay system which is highly sensitive and which is homogeneous in nature.

U.S. Patent 3,939,350 to Kronick and the Kronick citations therein referenced describe an immunoassay system which allows for the measurement of biochemical analytes by fluorescence in a liquid sample. Kronick employs a physical phenomenon known under the name of total internal reflectance. This optical phenomenon occurs wherein light, when directed through a high refractive index material toward the interface of that material with a second material having a lower refractive index, at greater than a critical angle, all light is reflected from that interface save for a microscopic evanescent wave which propagates into the second material for only a short distance. The second material may, for instance, be water or other aqueous medium in which an assay is being conducted. Kronick noted that when he brought materials which had been fluorescently labeled down to the interface and within the field of the evanescent wave, he could energize the fluorescent molecules and detect fluorescence which then emanated into the overlying solution. The Kronick system, however, looks at fluorescence which cannot be readily modified by alteration of the fluorescent labels in order to suit the system under study. Due to the nature of the specificity of the fluorescent label with respect to the wavelength of the excitation frequency, one is limited to a discrete light source providing the critical excitation frequency. To date, most investigators favor the He-Ne laser light source due to its reliability and low cost as well as the low cost of associated optics. Such a light source, however, brings concomitant difficulties in tailoring fluorescent molecules to be excited by the He-Ne laser output. The organic, inorganic, and bio-organic techniques required are especially difficult to control in the immunoassay arena. Further, Kronick's reliance on fluorescence is accompanied by additional disadvantages associated with bleaching of the fluorescent molecules and generally critical matching of fluorescent molecule excitation wavelength with laser output wavelength necessary to obtain good quantum efficiency.

It is an object of the present invention to provide a new immunoassay system which avoids the disadvantages associated with fluorescent labels and the criticality associated with matching an excitation source.

It is another object of the present invention to employ the principles of total internal reflection but with far greater flexibility regarding the choice of illumination sources.

U.S. Patent 4,181,441 Noller describes a system similar to that of Kronick. Noller, however, taught that the assay should be conducted by measurement of light absorption in a liquid sample which could then be correlated to the presence of biochemical analytes. Although the Noller system employs different physical principles than the Kronick system, light absorption measurements are similarly subject to poorer signal-to-noise ratios due to small differences in large light signals thereby making such a system inherently less sensitive than desired.

It is another object of the present invention to avoid employing light absorption measurements while still gaining the advantages to be provided by the total internal reflectance phenomenon.

U.S. Patent 4,521,522 to Lundstrom teaches yet another immunoassay based upon reflectance and the use of Brewster's angle. This system relies upon a different optical phenomenon wherein directing a light beam, polarized in the plane of incidence, upon an interface, for example that formed between plastic and liquid, results in the transmission of a strong light beam into the liquid when such light strikes the interface at the Brewster angle. At the Brewster angle, substantially no light is reflected.

The Brewster angle is a function of the refractive indices of the two materials as well as the direction of polarization. Lundstrom noted that upon the growth of a biochemical layer at the interface, the Brewster angle condition would be disrupted resulting in increasing light reflectance, particularly at angles less than the Brewster angle. Unfortunately, the Lundstrom assay only works effectively with a wash step since the transmission of the beam into the liquid also results in the generation of light scatter and thus a spurious signal.

It is another object of the present invention to utilize light scatter but to avoid light scatter generated by the transmission of light into the liquid which occurs naturally when light is directed at an interface at the Brewster angle. Accordingly, it is yet another object of the present invention to avoid employing a Brewster angle condition.

### Summary of the Invention

In accordance with various aspects and the principles of the present invention, there is provided an immunoassay system which utilizes scattered total internal reflectance (STIR) as a measure of the presence of particular ligands to be determined in an aqueous solution. The invention relies in part upon the identification of the critical angle associated with total internal reflectance. The angle is largely a function of the refractive index of the material through which an incident light wave is directed, e.g. plastic, and the relatively lower refractive index of the material in which the immunoassay is being conducted, e.g. an aqueous solution. It is measured from a line perpendicular to the interface between the two materials, and thus at its maximum,  $90^\circ$ , will lie in the plane of the interface.

Light directed through the plastic toward the interface formed by the aqueous sample and plastic materials at the critical angle will result in total internal reflectance of the light within the plastic. It is recognized that no materials in the real world are perfect and accordingly, it is preferred that the incident light be directed toward the interface at an angle several degrees greater than the critical angle, most preferably in the range of approximately  $6^\circ$  greater in order to ensure that the basic conditions of total internal reflectance are met. At such an angle, the incident collimated light, preferably from a laser, is totally internally reflected within the plastic save for the propagation of the evanescent wave parallel to the surface of the plastic and approximately  $1/4\lambda$ , from the surface. Similarly, smooth surfaces at the interface are preferred for optimum signal quality. Unlike conventional fluorescent techniques including those of Kronick, the present assay system is flexible with respect to light wavelength since particle size may be readily adjusted to match the available light source (or vice versa) to provide acceptable light scatter. Fluorescent molecules are not readily adjustable with respect to excitation wavelength.

Most ideally, the light source will be a He-Ne light source, however, other lasers with different wavelength outputs have been used and still other sources suggest themselves including light emitting diodes and other nonlaser light sources.

Applicant's immunoassay system further relies upon conventional immunoassay techniques but with one important difference. Applicant ideally employs a particulate label having a higher refractive index than that of the solution and most preferably also higher than the first light transmissive material, e.g. plastic in the foregoing example. Such particles would include, for instance, red blood cells, other materials having a highly reflective surface such as metallic particles, and nonmetallic substances such as glass or plastics, e.g. latex particles, and the like. Most preferably, colloidal gold is used as a label for the solution phase immunologically active component. While colloidal gold as a label is known, see for example U.S. 4,313,734 to Leuvering, almost no nonagglutination related use of the label has been made to date due to the

difficulties associated with detection, particularly in homogenous type systems. It was surprisingly discovered by the inventors hereof that the unique combination of STIR with colloidal gold has resulted in an extremely efficient and sensitive homogeneous assay system. It is believed, but not known for certain that this is due primarily to the interaction of the colloidal gold particles with the evanescent wave. Indeed, experience implies that particles having an increasingly higher index of refraction than that of the underlying solid generally increasingly scatter light. While particles with indices of refraction less than the underlying solid, providing they are also not equal to that of the aqueous medium, would also scatter light, such are less preferred.

Assuming for the moment a conventional sandwich technique, one immunoglobulin or ligand binding partner is immobilized on the surface and binds antigen or other ligand to be determined. Thereafter, (or simultaneously, or if not previously) a second immunoglobulin, directed at a second epitopic site on the ligand, and labeled directly or indirectly with colloidal gold, binds to the ligand creating the so-called "sandwich". In this arrangement, the presence of the colloidal gold disrupts the propagation of the evanescent wave resulting in scattered light which may be detected by a photomultiplier or other light sensor to provide a responsive signal. Another important aspect of the present invention involves the physical location of the detector. The detector is ideally placed at an angle greater than the critical angle and in a location whereby only light scattered backward toward the light source is detected. This location thereby ideally avoids the detection of spurious scattered light within the bulk liquid medium.

Another feature of the instant invention is that the immunoassays are diffusion rate controlled and not particularly temperature dependent. This is in strong contrast to ELISA and various other immunoassay techniques wherein temperature control is critical since small changes in temperature in such systems results in wide variations in assay results per unit of time.

It was surprisingly found by the inventors hereof, that as a result of the combination of these elements, rapid, sensitive results could be obtained in a homogeneous environment without requiring the complicated equipment previously associated with colloidal gold assay techniques.

#### Detailed Description of the Invention and Best Mode

An apparatus embodying the principles of STIR was constructed utilizing an equilateral flint glass prism, Model 01-PES-007, obtained from Melles-Grio. The prism was mounted on a support with one side held horizontal. An antibody-coated cuvette in the form of a microtiter well, available from Dynatech under the trade name IMMUNOLON TWO (styrene) was optically coupled to the horizontal prism surface with standard microscope oil. A five miliwatt helium neon laser (Hughes 3225-H-PC) was used to illuminate part of the cuvette's bottom surface at an angle  $6^\circ$  past the critical angle. Optionally, a cylindrical lens may be used to assist in focusing the laser light beam.

The critical angle was first determined by filling an uncoated cuvette, optically mounted on the prism, with a scattering aqueous medium comprising a mixture of colloidal gold sol produced pursuant to the method reported in Scanning Electron Microscopy Vol II, 9-31 (1981). Sear Inc., AMF, O'Hare, Chicago, generating particle sizes of about 30 to 50 nm and serum. The prism was rotated along an axis transverse to the axis of the incident light until the laser beam path, visible inside the cuvette, optically disappeared indicating that substantially all of the incident light was being reflected at the cuvette-liquid interface, the internal reflectance phenomenon known to occur at the critical angle. This critical angle between a perpendicular through the surface having the optically mounted cuvette and the laser beam was measured, the prism was reinstalled to provide a horizontal surface and the laser adjusted to illuminate the surface internally through the prism at an angle equal to  $6^\circ$  plus the critical angle. While a polarized laser was used with its polarization aligned with the electric field parallel to the plane of the styrene liquid interface, such is merely preferred but not necessary. Indeed virtually any collimated illumination source will serve. Similarly, while a prism was convenient, any optical coupling device for directing illumination toward the aqueous solid interface may be used such that total internal reflectance can be achieved by that interface.

A photodetector (Hamamatsu No. G1742 photodiode) was positioned at an angle above the critical angle but less than  $90^\circ$  at a position physically near the laser such that it would detect light scattered back toward the laser. In this position, minimal laser light is detected prior to the assay despite imperfections present at the interface.

Thus, placement of the photodetector above the critical angle is believed very important in order to insure that light propagating through the solution, e.g. straight light or secondary light scatter induced by irrelevant sources, cannot reach the detector. As a related advantage, this greatly reduces the effect of the sample's color or turbidity and of bubbles present at the liquid interface.

The electrical signal from the photodetector was electrically coupled to a high gain current amplifier (Kiethly Electrometer 610-C) and the output recorded on a strip chart recorder or digitally recorded by a computer data acquisition system (HP controller 3497A with HP 9836 computer). Reaction rates were then graphically determined on the recorder chart or calculated conventionally employing the computer.

#### Example 1 - hCG Sandwich Assay

An anti-hCG antibody-coated cuvette (coated by standard physical adsorption) was positioned on the oil-coated prism with a laser internally reflecting off the center of the cuvette. 35  $\mu$ ls of assay buffer (0.01 M phosphate buffered saline at a PH of 7.4 containing 1% bovine serum albumin, 1 M NaCl and 1.5 mg/ml mouse IgG) was added to the cuvette. 50  $\mu$ ls of nonblocking, anti-hCG antibody coupled with colloidal gold (approximately 44 nm in size) was then added and mixed by pipette aspiration. 25  $\mu$ ls of serum sample or serum-based standard (Gilford) was then added to the cuvette and mixed. The intensity of the scatter signal was recorded by a strip chart recorder and by a digital data acquisition system. The reaction rate was permitted to equilibrate for the first five minutes to permit the system to become linear and then measured kinetically during the next five minutes. Reaction rates (e.g. signal slopes) of unknown serum samples were compared to the reaction rates of standards in order to compute hCG concentrations. The results were as follows:

<u>Std.</u>	<u>Signal Slope (arbitrary units)</u>
0 mIU	1.00
10 mIU	7.43
25 mIU	16.33
50 mIU	32.03
100 mIU	68.67
200 mIU	130.97

#### Example 2 -Test For Antibody (Reverse hCG Sandwich Assay)

hCG antigen was coated onto Immunolon cuvettes and positioned on the oil-coated prism as in Example 1. 50  $\mu$ ls of colloidal gold (approximately 45 nm) coated with hCG was added to the cuvette along with 35  $\mu$ ls of assay buffer as described in Example 1, and mixed. 25  $\mu$ ls of mouse monoclonal anti-hCG containing standard (diluted in pH 8.3 HEPES/TRIS 0.225 M +0.5% BSA) added and mixed. After a five-minute delay for equilibration, the rate was measured as in Example 1. As anti-hCG concentrations were increased up to 10  $\mu$ gs per ml, increasing rates of light scatter were observed with rates decreasing above this concentration giving the expected hook effect (e.g. insufficient labeled and immobilized antigen to accomodate all of the antibody present). The data was:

	<u>Mouse IgG Conc. (ng)</u>	<u>Signal Slope (arbitrary units)</u>
5	0 ng	8.02
	10 ng	10.27
10	100 ng	12.35
	1 $\mu$ g	75.84
	10 $\mu$ g	91.39
15	100 $\mu$ g	37.00

### Example 3 -Competition With Antigen-Coated Cuvette

Thyroxin ( $T_4$ ) was covalently coupled to BSA with approximately 20  $T_4$  molecules per BSA molecule employing the following procedure.  $T_4$ -BSA conjugate was prepared from coupling BSA with  $T_4$ MC, L-Thyroxinyl-4-(N-maleimido-methyl)-cyclohexane-1-carbonate, through a nucleophilic addition at pH 9-10 by amino groups of BSA to the maleimido group of  $T_4$ MC.  $T_4$ MC was derivatized from SMCC, succinimidyl-4-(N-maleimido-methyl)-cyclohexane-1-carboxylate (Pierce Chemical), with L-Thyroxine by amidation at neutral pH.  $T_4$ -BSA conjugate was absorbed to commercial, strip microtiter wells by incubating 0.1 mls of 0.17 mgs per ml of the conjugate in 0.01 M phosphate buffer adjusted to a pH of 7 at room temperature for 18 hours. The wells were washed three times with 0.25 M HEPES/TRIS buffer containing 0.05%  $NaN_3$ . 0.15 M NaCl at a pH of 8.3). The wells were then incubated for 72 hours at room temperature with 0.2 mls of HEPES/TRIS buffer plus 1% BSA. The wells were then again washed three times with HEPES/TRIS buffer and stored with buffer at 4°C. until use.

Colloidal gold having an average diameter of 40 nm was coated with monoclonal anti- $T_4$  IgG by previously described methods. The strip well cuvettes were mounted on the prism as in Example 1 and 65  $\mu$ ls of pH 7.4 PBS containing 0.02%  $NaN_3$  and 2% bovine gammaglobulin was added to the cuvette followed by 10  $\mu$ ls of  $T_4$  standard in the same buffer. 25  $\mu$ ls of anti- $T_4$  antibody coated colloidal gold was then added to the cuvette and mixed. The reaction rate was measured after an equilibration period. As expected, increasing  $T_4$  concentrations correlated with decreased signal rates from back scattered light signal as follows:

	<u><math>T_4</math> - (<math>\mu</math>g/dl)</u>	<u>Signal Slope (arbitrary units)</u>
40	0	51.1
	2	41.9
	4	25.3
	8	9.08
50	12	6.51
	24	2.96

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#### Example 4 -Competition With Antigen-Coated Colloidal Gold

Immunolon strip well cuvettes were coated with 0.1 ml of 5  $\mu$ g/ml of anti-digoxin and 0.1 M  $\text{K}_2\text{PO}_4$  at a pH 7.4 and stored at 4°C. until use. The wells were then washed three times with 0.01 M PBS at a pH 7.4. Colloidal gold particles having an average diameter of 40 nm were coated with 1 mg per ml of digoxin-BSA conjugate (approximately 5 digoxins per BSA molecule) by the method set forth in an article by T. W. Smith, Biochemistry 9:331-337 (1970) and then diluted 1 to 4. 35  $\mu$ l of buffer (0.01 M PBS, 1.0 M NaCl, 1% BSA at pH 7.6) was added to the cuvette followed rapidly by the addition of 25  $\mu$ l of serum samples or serum base standard and 50  $\mu$ l of digoxin-coated colloidal gold suspension and mixed. The reaction rate was measured during the next five minutes and the results observed. Increasing digoxin concentrations resulted in reduced reaction rates as follows:

15	<u>Digoxin (<math>\mu</math>/ml)</u>	<u>Signal Slope (arbitrary units, 2 runs)</u>
	0	372, 296
	0.25	127, 86
20	0.50	30, 29

#### Example 5 -Internalized Kinetic Calibrator

It will be recognized that there may be variation from well to well between assays as well as between liquid reagents added to the wells. These differences will result in variations in kinetic responses which could, without correction, lead to erroneous results. One preferred method of correction is to utilize an internalized kinetic calibrator. To do so, a low level control sample is added to the well at the beginning of every assay and the rate of reaction monitored for a short time prior to the addition of the sample to the same well. The control sample can thus be used to calibrate each individual well, e.g. measuring the well's sensitivity and using that information to correct the sample readings, thereby obviating differences in structural or reagent coating uniformity. Accordingly, homogeneous rate assays can be ideally performed by first adding a control sample and monitoring the level of detector output. As a related advantage, this procedure will eliminate the need to perform duplicate assays thereby saving in time and resource expenditures. Such a calibration procedure will also obviate the sample to sample variations in light scattering efficiency of the particles which is a strong function of the index by refraction of the individual sample. The following example of the procedure demonstrates the principles involved.

Molded polycarbonate cuvettes were adsorbed coated with anti-hCG antibody. 150  $\mu$ l of assay buffer (from Example 1). 100  $\mu$ l of anti-hCG coated colloidal gold (approximately 40 nm diameter, and 75  $\mu$ l of Gilford stripped serum based 10 mIU/ml calibrator was added to each cuvette and mixed. After a 5 minute incubation, the rate of increase of scattered light intensity (slope) was measured during the next 5 minutes. After recording this calibration slope, 75  $\mu$ l of Gilford serum based standard was added as sample, mixed and incubated 5 minutes before reading the scattered light slope during the next 5 minutes. The net calibrated slope of each cuvette was calculated by the equation:

$$\text{Net calibrated slope} = [\text{slope of standard/slope of calibrator}] - 0.8826$$

Where 0.8826 was the average slope of six zero hCG standards divided by their respective calibration slopes.

The CV (coefficient of variation) of six replicates of the following standards were calculated on the basis of the net calibrated slope and compared to the uncorrected slope of these standards. The data was as follows:

	mIU/ml of standard	CV of uncorrected	CV of Net Calibrated slope
5	10 mIU/ml	18.31%	10.79%
	50 mIU/ml	30.3 %	21.42%
10	100 mIU/ml	18.86%	5.88%
	200 mIU/ml	33.63%	30.86%

In all cases, it can be seen that greater accuracy and repeatability was obtained using the internal calibration method.

#### Example 6 - Competitive hCG Assay Using Latex Particles

Immulon strip wells were coated as stated in Example 1 above. 35  $\mu$ ls of assay buffer was added to each well. 25  $\mu$ ls of hCG dissolved in stripped serum (Gilford) was then added and mixed. After a 5 minute incubation, 50  $\mu$ ls of "Ortho Beta-hCG Slide Test for Pregnancy" hCG coated styrene latex (0.375 micron diameter) (Ortho Diagnostic Systems Inc.) was added and mixed. The reaction rate was permitted to equilibrate for 5 minutes while the slope of the scattered light signal was calculated during the next 5 minutes. The results were as follows:

	HCG Standard Concentration	Signal Slope (arbitrary units)	Average
30	223,875 mIU/ml	3.61, 3.76, 6.04	4.47
	22,387	8.96, 9.02, 9.25	9.08
35	2,238	118, 122, 144	128
	223	158, 162, 187	169
	22.3	148, 157, 196	167
40	2.2	138, 142, 161	147

#### Example 7 -Direct Red Cell Antigen Test using Red Cell Particle (approximately 8 micron diameter)

Polycarbonate cuvettes were coated by adsorption with anti-D (anti-Rh<sub>0</sub>) for an RH factor test and with anti-A for an ABO blood group test. 0.5 ml of human whole blood was centrifuged, resuspended in 5 ml of phosphate buffered saline (PBS) pH 7.4, centrifuged and resuspended in 2 ml of PBS. 300  $\mu$ ls of this sample suspension was added to the coated cuvette and mixed. After a 2 minute incubation, the slope of the scattered light intensity was calculated over the next 8 or 18 minutes. The results were as follows:



## Slope in anti-A Coated Cuvettes

## 5 Red Cell Phenotype

	Sample Blood Type (RH Type)	Slope (Time)
10	A-	267 ( 8 min)
	A+	240 (18 min)
	B+	-18.6 ( 8 min)
15	O	14.9 (18 min)

## Slope in anti-D Coated Cuvettes

20	Sample Blood Type (RH Type)	Slope (Time)
	A+	56.6 (18 min)
25	B+	10.2 (18 min)
	O-D-(high positive RH)*	32.3 (18 min)
	A-	4.3 (18 min)
30	O-	4.5 (18 min)

## \*rare blood type

It will be readily recognized by those skilled in the art that a certain amount of physical manipulation may be made to this system without substantially departing from either the spirit or the scope of the present invention. For example, the cuvettes and prism assembly may be one integral unit wherein the cuvette microtiter well is molded with a plastic prism forming part of the cuvette. Similarly, while an angle 6° above the critical angle has been found most preferred, it will be recognized that dependent upon the optical characteristics of the illumination source and the photodetector, certain variations above the critical angle may be more optimal and are to be deemed equivalent to the angle set forth herein. Further, measurements may take place on the side or bottom of the cuvette.

Further, while colloidal particles such as gold, latex and red blood cells have been described in the Examples, it should be recognized that particles and their particular size range are not to be deemed limitations but are merely exemplary of the wide range of possibilities. Indeed, the size of particles generally should be chosen with consideration given to the wavelength of the light in the liquid medium (in turn a function of the refractive index of the medium), the index of refraction of the particle should ideally be chosen with consideration given to the index of refractions of the aqueous medium and the solid so that the net effect is an optimum signal, most advantageously obtained when resonance of the system occurs. While predictability is exceedingly difficult given the current level of understanding of these complicated interactions, the actual optimization procedures are relatively simple and easily performed by those skilled in the art.

## Claims

1. Apparatus for use in an immunoassay system employing a light scattering particle labeled immunologically active component for the detection of a ligand or ligand binding partner in an aqueous solution comprising;

a container for receiving the aqueous sample said container having a refractive index greater than that of the aqueous sample,

- 5 a light source for providing illumination,

optical coupling means for directing said illumination toward the interface between the container and the aqueous solution,

- 10 alignment means for receiving said cuvette in fixed relationship to said optical means and said light source whereby said illumination is directed toward the interface between the container and the aqueous solution at an angle equal to or greater than the critical angle, and

- photodetector means for receiving light scattered above said critical angle by said light scattering particle  
15 and back toward said light source.

2. The apparatus of Claim 1 wherein said light source provides collimated illumination and said optical coupling means is a prism which is optically coupled to said container.

3. The apparatus of claim 1 or claim 2 wherein said light source is a laser.

4. In an immunoassay system for the detection of ligands or ligand binding partners in an aqueous  
20 solution, the improvement comprising employing colloidal gold as a label in said immunoassay, conducting said immunoassay in the field of an evanescent wave, measuring light scattered by the presence of said colloidal gold particle in said evanescent field and correlating said colloidal gold scattered light as a function of the presence of the ligand or ligand binding partner to be determined.

5. A method for determining the presence of a ligand in an aqueous sample suspected of containing  
25 said ligand comprising the steps of:

providing a first ligand binding partner specific for said ligand in said aqueous sample, said first ligand binding partner being labeled with a particle having light scattering characteristics in said aqueous sample;

- 30 combining said first ligand binding partner and said aqueous sample with one side of an optically transmissive material thereby forming an interface between said material and said sample, said material having a plurality of second ligand binding partners bound to said material in contact with said aqueous sample and capable of binding to said ligand, said optically transmissive material having a refractive index greater than the refractive index of said aqueous sample;

- 35 illuminating said interface through said optically transmissive material at an angle to said material to provide total internal reflectance within said material; and measuring the amount of light scattered from said interface, wherein said amount of scattered light is a function of the amount of ligand present in said assay medium.

- 40 6. The method as provided in claim 5 wherein said measuring step is conducted at an angle greater than said critical angle and wherein substantially only back scattered light is detected.

7. The method according to claim 5 or claim 6 wherein said ligand binding partners are antibodies of monoclonal or polyclonal origin and said ligands react specifically therewith.

8. The method according to claim 5 or claim 6 wherein said ligand in said sample to be determined is  
45 an antibody and said second ligand binding partners bound to said material are antigens or haptens specifically reactive with said sample antibodies.

9. A method as provided in any one of claims 5 to 8 wherein said particles are selected from the group consisting of colloidal gold, latex particles, glass particles, metallic particles, nonmetallic particles and red blood cells.

- 50 10. A method as provided in any one of claims 5 to 9 wherein said illuminating step comprises irradiating said surface with light from a helium-neon laser.

11. A method as provided in any one of claims 5 to 10 wherein said first ligand binding partner is added to said optically transmissive material prior to adding said aqueous sample.

12. A method as provided in any one of claims 5 to 10 wherein said aqueous sample is added to said  
55 optically transmissive material prior to adding said first ligand binding partner.

13. A method as provided in any one of claims 5 to 10 wherein said aqueous sample and said first ligand binding partner are mixed prior to combining with said optically transmissive material.

14. A method as provided in any one of claims 5 to 13 wherein said particle is colloidal gold.

15. A method as provided in any one of claims 5 to 14 wherein said measurement step is performed a plurality of times to derive a rate which may be correlated to the presence of ligand in said aqueous sample.

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Europäische Patentamt  
European Patent Office  
Office européen des brevets

(11)

Publication number:

**0 254 430  
A3**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: **87305669.1**

(22) Date of filing: **25.06.87**

(51)

Int. Cl.4: **G 01 N 33/543**

**G 01 N 21/47**

**// G01N33/557, G01N33/76,  
G01N33/78, G01N33/94,  
G01N33/80**

(30) Priority: **26.06.86 US 879236**

(43) Date of publication of application:  
**27.01.88 Bulletin 88/04**

(84) Designated Contracting States:  
**AT BE CH DE FR GB IT LI LU NL SE**

(88) Date of deferred publication of search report:  
**05.10.88 Bulletin 88/40**

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(54) **Light-scattering immunoassay system.**

(57) A new immunoassay system is provided for the detection of ligands or ligand binding partners in solution in a heterogeneous format. The invention relies upon the detection of back scattered light from an evanescent wave disturbed by the presence of a colloidal gold label brought to the interface by an immunological reaction. The evanescent wave existing at the interface in turn is the result of a totally internally reflected incident light wave. Placement of the detector at a back angle above the critical angle insures a superior signal-to-noise ratio.

**EP 0 254 430 A3**



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

EP 87 30 5669

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	EP-A-0 170 376 (UNILEVER PLC) * page 4, line 12 - page 5, line 2; page 5, line 2 - page 6, line 10; page 6, line 29 - page 7, line 3; page 8, line 8-19; page 9, lines 13-23; page 10, line 32 - page 11, line 5; page 12, lines 14-24; claims 1-15 *	1,3	G 01 N 33/543 G 01 N 21/47 G 01 N 33/557 G 01 N 33/76 G 01 N 33/78 G 01 N 33/80 G 01 N 33/94
A	---	5,6	
X	EP-A-0 075 353 (BATELLE MEMORIAL INSTITUTE) * page 14, lines 25-28; page 25, line 25 - page 27, line 3; examples 1-3; figures 10a,b + 13a,b; claims 1-7,10 *	1	
A	EP-A-0 753 535	3, 5-8, 10-13, 15	
X	EP-A-0 167 335 (D.F. NICOLI et al.) * page 19, line 14 - page 20, line 13; page 23, lines 13-14; page 31, lines 16-18; page 32, line 8 - page 33, line 4; page 33, line 24 - page 34, line 6; page 34, lines 11-14 - page 38, lines 21-24; page 1, line 10 - page 42, line 11; figure 1; claims 1-14 *	1	TECHNICAL FIELDS SEARCHED (Int. Cl. 4)  G 01 N 33/00 G 01 N 21/00
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A,D	US-A-3 939 350 (M.N. KRONICK et al.) * column 8, lines 53-54; claims 1-3, 10-13 *	1-3,5-7	
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The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 04-07-1988	Examiner DE KOK A.J.
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document  T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document			

EPO FORM 1503 03.82 (P/801)



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	JOURNAL OF CHROMATOGRAPHY, vol. 376, April 1986, pages 175-189, Amsterdam, NL; C.J. GRIBNAU et al.: "Particle-labelled immunoassays: A review" pages 175-182 ---	1,4,9,14	
A	EP-A-0 091 636 (WAKO PURE CHEMICAL INDUSTRIES LTD.) * page 11, line 24 - page 12, line 6; claim 1; figure 1 * -----	1,3,15	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 04-07-1988	Examiner DE KOK A.J.
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document			

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